Temperature sensitivity of human perforin mutants unmasks subtotal loss of cytotoxicity, delayed FHL, and a predisposition to cancer

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The pore-forming protein perforin is critical for defense against many human pathogens and for preventing a catastrophic collapse of immune homeostasis, manifested in infancy as Type 2 familial hemophagocytic lymphohistiocytosis (FHL). However, no evidence has yet linked defective perforin cytotoxicity with cancer susceptibility in humans. Here, we examined perforin function in every patient reported in the literature who lived to at least 10 years of age without developing FHL despite inheriting mutations in both of their perforin (*PRF1*) alleles. Our analysis showed that almost 50% of these patients developed at least 1 hematological malignancy in childhood or adolescence. The broad range of pathologies argued strongly against a common environmental or viral cause for the extraordinary cancer incidence. Functionally, what distinguished these patients was their inheritance of *PRF1* alleles encoding temperature-sensitive missense mutations. By contrast, truly null missense mutations with no rescue at the permissive temperature were associated with the more common severe presentation with FHL in early infancy. Our study provides the first mechanistic evidence for a link between defective perforin-mediated cytotoxicity and cancer susceptibility in humans and establishes the paradigm that temperature sensitivity of perforin function is a predictor of FHL severity.

hemophagocytic lymphohistiocytosis | immunodeficiency | leukemia | lymphoma

The granule-mediated, target-cell death pathway is executed by cytotoxic lymphocytes (CL) and driven by synergy between a pore-forming toxin, perforin (1) (PRF; *PRF1*) and proapoptotic serine proteases, granzymes. These cytotoxins are released into the synaptic cleft between CL [cytotoxic T lymphocytes (CTL) or natural killer cells (NK)] and target cells. PRF monomers assemble into ≈15–20-nm transmembrane pores in the target-cell, permitting granzymes to enter the cytosol of a virus-infected or transformed cell and initiate caspase-dependent and -independent cell death pathways (2). In humans, a critical role has been demonstrated for PRF in maintaining immune homeostasis in the first few months after birth (3). Thus, the inheritance of biallelic *PRF1* mutations is responsible for 30–60% of cases (4) of a rare autosomal recessive disorder, Type 2 familial hemophagocytic lymphohistiocytosis (FHL), affecting ≈1 in 90,000 live births (5–8). FHL is generally triggered by environmental or common microbial antigens and is almost always fatal within a few months of birth, unless treated with allogenic bone marrow transplantation (BMT) (9).

Recently, considerable evidence has emerged that the CLs of inbred mice can detect and destroy premalignant cells to delay the onset of cancer. Multiple studies have pointed out the heightened incidence of spontaneous hematological cancer in $PRF^{-/-}$ mice, their increased susceptibility to viral and chemical carcinogens, and their inability to reject transplanted tumors (10). The mouse immune system may also hold some malignancies in check for protracted periods so that a cancer and the immune response it evokes remain in a state of "equilibrium," with cancer finally becoming overt only when CLs become depleted or the tumor mutates and escapes immune control (11). Nonetheless, some investigators have questioned the pathophysiological relevance of the findings, pointing out that many of the tumors induced by chemicals arise rapidly and are strongly immunogenic, whereas other cancers can be explained by the failure to clear oncogenic viruses (12).

Amid the controversy in mice, some proponents of "cancer immune surveillance" have proposed that the human immune system also protects against spontaneously arising cancers. Unsurprisingly, this proposition also remains highly controversial, and the role of CLs in influencing the susceptibility to human cancer remains hotly debated. It is clearly problematic to extrapolate experimental data from inbred mouse strains to an outbred human setting where such evidence is far more difficult to gather. As a result, the only informative human studies have been associative, but never mechanistic (13–15).

Unlike other congenital immune deficiencies, PRF deficiency specifically and directly affects CL effector function with no other known impact within or outside the immune system. Therefore, demonstrating an association between inherited PRF dysfunction and human cancer susceptibility would strongly imply a role for CTL and/or NK cells in cancer immune surveillance. We observed that FHL can sometimes be greatly delayed in carriers of biallelic *PRF1* missense mutations (16). In the current study, we show that almost half of this group of children and young adults developed either leukemia or lymphoma. To explore the molecular basis for this relationship, we tested the mutated *PRF1* alleles for their cytotoxic function. Almost invariably, these mutants had no detectable function, but, remarkably, most were temperature-sensitive in that function was restored at reduced (permissive) temperature.

Our findings demonstrate a link between partial (subtotal) loss of PRF function, the delayed onset of FHL, and increased susceptibility to hematological malignancies. Our results also provide support for the hypothesis that, as in mice, the human immune system can mediate immune surveillance against transformed hematopoietic cells.

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Table 1. Patients with biallelic *PRF1* mutations presenting with delayed FHL (>10 years) or an alternative primary diagnosis

	PRF1		Primary disease		Manifestation of FHL		
Patient	Allele 1	Allele 2	Diagnoses	Age of onset, yrs	Age of onset, yrs	Outcome	Refs.
			Haematological malignancy				
1*	T435M	T450M	EBV-positive Hodgkin lymphoma; B cell non-Hodgkin lymphoma	7, 10	10	BMT	30
2*	G305D	R356W	T cell lymphoma	18	18	BMT	30
3*	A91V	F421C	T cell lymphoma	7	FHL(?),18	Died	30
4*	A91V	R232H	Anaplastic large cell lymphoma	13	No FHL	NR	31
5(s)	A91V	W374X	T-lymphoblastic lymphoma	21	No FHL	BMT	30, 32
6	A91V	A91V	Acute lymphoblastic leukemia	2	No FHL	NR	33
7	A91V	A91V	Chronic lymphoblastic leukemia	? (adult)	No FHL	NR	34
8–11	A91V	A91V	Acute lymphoblastic leukemia	? (child)	No FHL	NR	35
			Viral infections				
12*†	R356W	T450M	Post-EBV demyelination	16	18	Died	36
13*	F193L	R410P	T cell lymphoproliferation	7, 11	FHL(?),18	Died	37
			Type 2 FHL				
14*	P39H	G149S	FHL		10	NR	3
15*	P201T	K285del	FHL		10	NR	38
16*	H222Q	R232H	FHL		>10	NR	38
17*	G317R	M1V	FHL		11	BMT	39
18	R410W	FS	FHL		12	BMT	39
19*‡	(A91V + R232H)	A91V	FHL		13	Died	40
20–22	A91V	A91V	FHL		17, 22, 49	Died	34, 41
23(s)	A91V	W374X	FHL		22	Chemo. (alive)	32

(s), Siblings; FS, frame-shift or nonsense mutations resulting in PRF; NR, not recorded; Chemo., chemotherapy.
*Mutants analyzed in this study.
†An asymptomatic sibling with the same *PRF1* mutations underwent preemptive BMT at the age of 17 years.
‡An asymptomatic fraternal twin was healthy at the age of 13 years.

Results and Discussion

Choosing a Study Population. Given the well-recognized association of PRF deficiency and spontaneous hematological malignancy in mice, we wished to explore whether a similar association exists in humans. Our study posed a significant methodological problem, as *PRF1*-associated Type 2 FHL is a rare disease and, apart from the relatively common A91V mutation, sporadic, monoallelic *PRF1* mutations affect only ≈1 in 400 individuals. PRF-deficient humans generally succumb to overwhelming FHL in early infancy and die unless they undergo heterologous BMT. It thus became apparent at the outset that a classic population-based approach would not be feasible in humans and an alternative was required. Since the initial finding in 1999 that biallelic *PRF1* mutations cause FHL (3), ≈200 such cases have been described. Of these 200 individuals, only 61 patients inherited at least 1 allele with a missense *PRF1* mutation. We noted that a proportion of these patients also presented with FHL much later in life (16). Although the functional basis for this delay has never been explored, we hypothesized that a subtotal loss of PRF function might be responsible. In principle, investigating this patient cohort might also provide insights into PRF function during adult life, including a putative role in cancer prevention.

On this basis, we identified a subgroup of individuals from nonconsanguineous families who possessed 2 mutated *PRF1* alleles but whose onset of FHL was markedly delayed (the age at onset of 10 years or older) or even abolished. A total of only 23 such cases could be identified in the entire literature (Table 1). We found that all 23 cases were from the subgroup of 61 individuals who had inherited at least 1 missense mutation. Ten of the individuals (Patients 14–23 in Table 1) developed manifestations of FHL without any other significant infectious or neoplastic sequelae reported. However, the remaining 13 individuals (≈56% of the cohort) presented with a primary life-threatening illness other than FHL (Patients 1–13 in Table 1), and only some have later developed hemophagocytic lymphohistiocytosis. Remarkably, in 11 of these 13 individuals (or 48% of the entire cohort of 23), the primary clinical presentation was with either B or T cell lymphoma or acute or chronic leukemia of lymphoid origin. This range of malignant pathologies and the patients' geographic spread largely excludes the likelihood that a single exogenous factor, such as exposure to an oncogenic virus, accounts for these malignancies. Apart from Patient 1, one of whose two separate malignancies was Epstein–Barr virus (EBV)-associated Hodgkin lymphoma, the remaining cancers are not known to be triggered by viruses.

The very high frequency of hematological cancers in this 23-patient cohort (11 of 23 patients, or 48%), or even as a proportion of all known cases of FHL where at least 1 missense mutation was inherited (11 of 61 patients, or 18%) is vastly in excess of that in the general population. The Surveillance, Epidemiology and End Results (SEER) Program estimates the lifetime incidence of all hematological cancers as 3.5%, comprising a 2.24% incidence of lymphoma and a 1.26% risk of leukemia (17). Even adopting the conservative 18% incidence of hematological malignancy observed in our extended cohort of 61 patients, this cancer incidence was far in excess of the number of expected cases ($P < 0.01$; Fisher Exact Test). It was also notable that all 11 of our cancer cases had their disease onset before the age of 20 years (Table 1). The incidence of cancer in our cohort of patients also greatly exceeds what has been reported for other primary immune deficiencies (<10%, ref. 18). Whereas immune-suppressed transplant recipients have a markedly increased incidence of virus-associated malignancies, their risk of nonvirus-associated cancer is also far lower, typically 1.2- to 2.5-times that of the general population.

We postulated that an analysis of *PRF1* mutations identified in patients with a paradoxically delayed onset of FHL and an unusually high incidence of cancer (Table 1) would potentially establish a functional link between PRF (or CLs) and immune surveillance in humans. We hypothesized that some defective *PRF1* alleles might provide sufficient PRF activity to enable escape from overwhelming FHL in infancy, but simultaneously unmask a susceptibility to malignancy in later years.

Reconstituting the Function of Late Onset FHL-Associated PRF1 Mutations in PRF1-KO CTLs. We therefore went on to examine the function of all 17 missense mutations (Table 1) using primary CTLs

derived from *PRF1*-KO mice whose $CD8^+$ T cells also expressed a clonotypic T cell receptor (OT-1) that recognizes the ovalbumin peptide SIINFEKL. We have already extensively tested and validated this methodology to demonstrate that the common PRF variant hA91V results in a partial loss of PRF cytotoxicity (19), and therefore some of the functional assays with this allele have not been reported again in the current study. The *A91V* allele has been found in up to 9% of individuals in several population surveys. We recently showed that purified recombinant hA91V-PRF has severely impaired lytic function when applied directly to nucleated or nonnucleated target cells [<10% of human wild-type (hWT) PRF activity] (19); however expression in *PRF1*-KO CTL improved its function to ≈50% of WT (19). Twelve individuals in the current study (7 of 11 that had cancer and 5 of the 10 who developed delayed FHL only), were either homozygous for hA91V-PRF or inherited it together with a completely inactive allele encoding a nonsense or frame mutation (Table 1).

We then turned to the remaining 16 missense mutations (excluding M1V, which abolishes protein translation), and tested their effect on PRF cytotoxicity (Fig. S1). With the exception of hF421C and hR232H, all of the remaining mutations were detrimental for PRF function because they were incapable of restoring any cytotoxicity to *PRF1*-KO CTLs. Based on these observations, we predicted that Patients 1, 2, 12–15, 17, and 18 (Table 1) lacked any functional PRF. R232H-PRF had considerable residual activity (≈50% of hWT, see Fig. S1*B*) and the compound mutant hA91V+R232H displayed some measurable function (Fig. S1*E*; ref. 20).

F421C-PRF, which was inherited by Patient 3, had normal activity in our assays (Fig. S1*B*). Given that this patient had apparently inherited 1 allele with WT activity, it is possible that this lymphoma was not associated with PRF dysfunction. Although speculative, it is also feasible that a further polymorphism in the noncoding regions of the hF421C mutant allele results in downregulated expression and therefore a relative excess of the less active, second allelic product, hA91V. Alternatively, significant dominant–negative activity has recently been demonstrated for A91V (19), and may have had a negative impact on the overall activity of Patient 3's CTLs. A dominant–negative interaction is also possible in Patient 4 (Table 1), who inherited the partially active mutants A91V and R232H, affecting different regions of PRF (Fig. S2).

A High Proportion of Patient Mutations Cluster to the Same Subdomain of PRF. Given the apparent complete loss of cytotoxic activity seen in the great majority of the *PRF1* alleles we tested (Fig. S1), it seemed puzzling that many of the patients (Patients 1, 2, 12–15, 17, and 18) had escaped FHL in infancy and in some cases, survived well for many years. To determine whether the mutations had any common feature, we first modeled their locations on the crystal structures of several PRF-like proteins that we and others solved recently (21–23). We were surprised to discover that the 4 mutations hA91V, hF193L, hP201T, and hR356W, present at least once in 18 of the 23 patients, were clustered within adjacent helices G and H of the MACPF domain. These helices were recently shown to play a critical role in PRF oligomerisation (24) despite being widely dispersed on the primary sequence (Fig. 1*A* and Fig. S2*A*). Our previous studies have shown that the reduced lytic function of hA91V PRF is at least partly due to protein misfolding (19), so we hypothesized that other disease-associated mutations mapping to the same vicinity might result in similar folding abnormalities.

Rescue of hF193L-, hP201T-, and hR356W-PRF Activity at Reduced Permissive Temperature. We noted recently that pore formation by PRF-like toxins relies heavily on major conformational changes because they function in an analogous fashion to the bacterial cholesterol-dependent cytolysins and undergo substantial rearrangement to form transmembrane pores (21–23). Molecules that

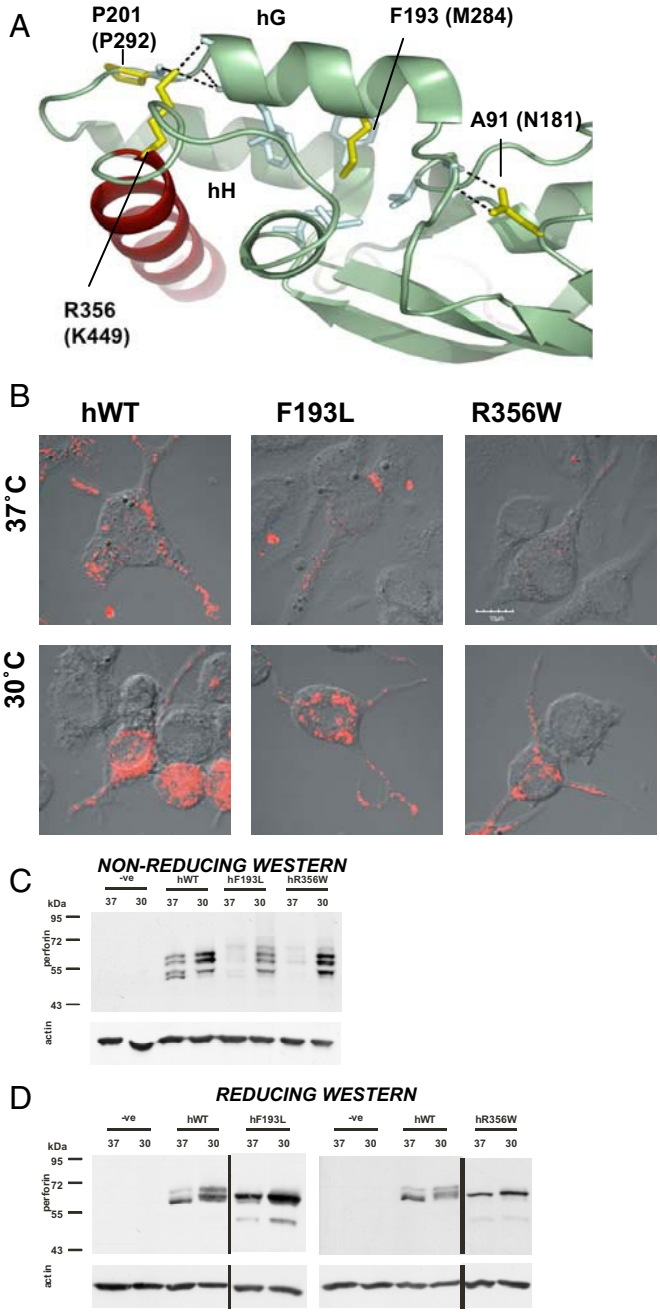


Fig. 1. Patient mutations that adversely affect protein folding, as demonstrated by loss of antigenicity. (*A*) Mapping PRF mutations on the predicted perforin structure. The position of the mutated perforin residues are mapped onto the structure of C8α with the residues equivalent to hA91, hF193, hP201, and hR356 shown in yellow stick and labeled. Numbering is for human perforin with C8α numbering and amino acid identifier shown in parentheses (details are in Fig. S2). (*B–D*) PRF-transfected RBL cells were grown for 18–24 h at 37 °C or at permissive temperature of 30 °C before immunofluorescence microscopy or immunoblotting. (*B*) Immunofluorescence microscopy of wild-type (hWT) and mutant PRF expressed in RBL cells shows the recovery of hF193L and hR356W expression at 30 °C compared with 37 °C as detected by anti-hPRF δG9. Only sorted GFP-expressing cells were assessed for PRF expression. (*C*) Nonreducing immunoblot using P1–8 demonstrates the loss of antigenicity of hF193L and hR356W mutant PRF as the mutants are essentially undetectable at 37 °C, whereas the signal is recovered at 30 °C. (*D*) Reducing immunoblot using P1–8 and the same samples as in *C* demonstrates the recovery of the PRF signal for mutants hF193L and hR356W both at 30 °C and 37 °C.

require marked conformational change for their function are often susceptible to mutations that impose an inappropriate conformation (25). We found that when the mutated proteins hF193L- and hR356W-PRF were expressed at 37 °C, they were virtually undetectable by immunofluorescence microscopy (monoclonal antibody δG9; Fig. 1*B*) and Western blotting under nonreducing conditions (monoclonal antibody P1–8; Fig. 1*C*). At first, we took this result to suggest that the PRF protein was degraded. However, when the very same samples were analyzed under more denaturing, reducing conditions that disrupted the 10 intrachain disulfide bonds of PRF, far stronger signals were seen (compare mutants at 37 °C in Fig. 1 *C* and *D*). This unexpected observation indicated that the mutants were not degraded, but rather displayed altered antigenicity under nonreducing (Fig. 1 *B* and *C*) and reducing (Fig. 1*D*) conditions at 37 °C. This change provided strong evidence that both mutations cause PRF to adopt a nonfunctional, misfolded conformation.

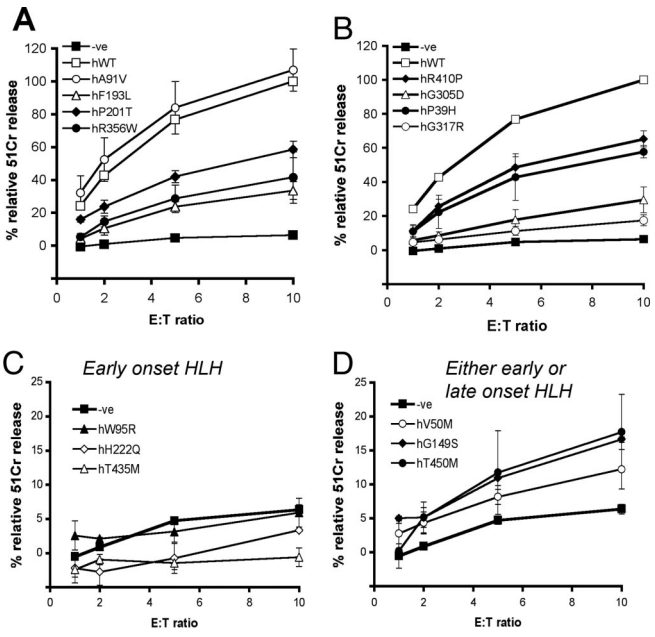
To test this possibility further, we expressed both hF193L- and hR356W-PRF under conditions that we predicted would minimize misfolding, for instance at a reduced (permissive) temperature. We therefore cultured PRF-expressing RBL-2H3 cells at 30 °C for 18–24 h after transfection. These conditions increased protein expression and, importantly, appeared to normalize their folding. Thus, a signal became obvious for each mutant, both by immunofluorescence microscopy (Fig. 1*B*, 30 °C) and Western immunoblotting under nonreducing conditions (Fig. 1*C*, 30 °C).

We then went on to test the activity of the mutants clustered to the same subdomain of PRF (F193L, P201T, and R356W) in the physiologically relevant context of *PRF1*-KO CTLs and at the permissive temperature of 30 °C. As expected, expressing hWT PRF in *PRF1*-KO CTLs at reduced temperature did not alter CTL cytotoxicity, and culturing primary human IL-2-stimulated NK cells at 30 °C also had no effect on their viability or cytotoxicity (Fig. S3). However, cytotoxic function of each of the mutants was restored to a significant level (compare Fig. S1 *D* and *F* and Fig. 2*A*), although it was still lower than that of hWT PRF-transfected *PRF1*-KO CTLs. Consistent with these results, the cytotoxicity of hA91V-PRF, which is partially active when expressed at 37 °C (19), was completely rescued by culturing the CTLs at 30 °C (Fig. 2*A*).

By using hP201T-PRF as an example, which (other than hA91V) had the highest level of recoverable activity (Fig. 2*A*), we found that adding the proteasome inhibitor MG-132 to reduce PRF degradation partially rescued hP201T PRF function at 37 °C (Fig. S4). However, MG-132 was considerably less effective at restoring function than culture at 30 °C (Fig. 2*A* and Fig. S4). Three other regulators of proteostasis (26) (indomethacin, celastrol, and 4-phenylbutyrate) were also tested, but did not have any significant effect on cytotoxic activity of hP201T. Taken together, our results suggest that correct mutant PRF folding, rather than simple correction of protein expression, was the key to rescuing mutant PRF and CTL cytotoxicity.

The Temperature Sensitivity of PRF Mutants Associated with Late-Onset FHL or Hematological Cancer Is a Generalized Phenomenon. We then went on to test all of the other mutants in the current study (Table 1) for their temperature sensitivity. Remarkably, the activity of virtually all of the mutants expressed by cancer or late-onset FHL patients was rescued to various degrees by prior culture of the CTLs at 30 °C (Fig. 2 *B* and *D*). The only exceptions were hT435M and hH222Q (Fig. 2*C*), but the second allele inherited by the carriers were partially active variants hT450M in Patient 1 (Fig. 2*D*) and hR232H in Patient 16 (see Fig. S1*B*), respectively. We were therefore able to conclude that all 23 patients in our study cohort possessed at least 1 allele with either some constitutive activity at 37 °C or the capacity to be functionally rescued at the permissive temperature. In short, none of these patients was truly null for PRF cytotoxicity.

The data presented above provided support for our hypothesis that inheritance of *PRF1* alleles that encode partially active PRF might enable patients to survive long enough without developing FHL to unmask their predisposition to other pathologies resulting from diminished PRF (and CTL) cytotoxicity. We reasoned that if this hypothesis is correct, then the converse should also apply, namely that alleles for which activity was not recoverable would be associated with early-onset FHL. We therefore tested the hW95R mutation because this allelic product is closely associated with early-onset FHL, and no cases of late-onset FHL have been reported in bearers of this mutation (3). hW95R-PRF was unable to complement the activity of *PRF1*-KO CTLs at 37 °C (Fig. S1*C*), nor was its function rescued with prior culture at 30 °C (Fig. 2*C*). Furthermore, we identified hH222Q and hT435M as the only 2 substitutions in our initial 23-patient cohort where cytotoxicity



Patient	*PRF1* allele 1	*PRF1* allele 2	Age of HLH onset
24	W95R	W95R	3 months
25, 26	H222Q	FS	1, 2 months
27	H222R	H222R	3 months
28	T435M	T435M	3 months

Patient	*PRF1* allele 1	*PRF1* allele 2	Age of HLH onset
29	V50M	FS	4 months
30, 31	V50M	V50M	7, 15 years
32	G149S	FS	2 months
33 - 38	G149S	G149S	1 ,3, 6 mo 1, 3, 10 yo
39, 40	R232H	FS	2, 12 years
41	T450M	T450M	7 years

Fig. 2. Temperature sensitivity of PRF mutants is a general phenomenon. (*A*) The activity of PRF mutants hA91V, hF193L, hP201T, and hR356W, which are all clustered to the same subdomain of PRF (Fig. 1*A*), is recovered at 30 °C (compared with 37 °C as shown in Fig. S1). Shown is the mean relative ^{51}Cr release ± SE of 3 independent experiments for each mutant and of 14 independent experiments for hWT and control (−ve). (*B*) PRF mutants hP39H, hG305D, hG317R, and hR410P demonstrate temperature sensitivity as their activity was recovered by culture at 30 °C. Shown is the mean relative ^{51}Cr release ± SE of 2–3 independent experiments for each mutant and of 14 independent experiments for hWT and control (−ve). (*C*) PRF mutants hW95R, hH222Q, and hT435M (early onset FHL) show no recoverable function at 30 °C. The *y* axis scale is increased and hWT not shown for clarity. Shown is the mean relative ^{51}Cr release ± SE of 2–3 independent experiments for each mutant and of 14 independent experiments for control (−ve). The table below shows Patients 24–28 identified in the literature, who inherited those *PRF1* mutations and all had early onset FHL. (*D*) PRF mutants hV50M, hG149S, and hT450M (either early- or late-onset FHL) recovered low, but significant, levels of activity. The *y* axis scale is increased and hWT not shown for clarity. Shown is the mean of relative ^{51}Cr release ± SE of 3–4 independent experiments and of 14 independent experiments for control (−ve). The table below shows Patients 29–41 identified in the literature, who had a variable (early or late) onset of the disease. FS, frame-shift mutations leading to premature stop-codons.

could not be recovered at 30 °C [defined as <5% specific ^{51}Cr release at an effector-to-target-cell (E/T) ratio of 10:1] (Fig. 2*C* and Fig. S1 *E* and *F*). We searched the literature once more and identified 5 cases in which hW95R, hH222Q, hH222R, or hT435M had been inherited in the homozygous state or as a single copy with a second frame-shift or nonsense (null) allele (Patients 24–28, detailed in Fig. 2*C*). In all of the reported cases, the onset of FHL was very early (before 3 months of age).

By contrast, we also identified several *PRF1* genotypes that occurred in either early- (<12 months of age) or late-onset FHL (Patients 29–41, Fig. 2*D*). Some of the patients with late-onset FHL were either from consanguineous marriages or could not have their nonconsanguinity verified; they were therefore excluded from the cohort in Table 1. Patients who inherited hV50M-, hG149S-, hR232H-, or hT450M-PRF as the only functional allele showed variable disease phenotype in that they succumbed to FHL at ages ranging from early infancy to teenage (detailed in Fig. 2*D*). Consistent with our prediction, these mutants showed complete loss of function following culture at 37 °C (Fig. S1) but had significant, yet relatively low, cytotoxic activity (defined as 10–20% of hWT PRF activity at an E/T ratio of 10:1; Fig. 2*D*) when cultured at 30 °C.

Compiling our data across all of the alleles tested in this study, there was a strikingly direct relationship between the age of onset of FHL (or other pathology) and the degree to which cytotoxic activity was recoverable at 30 °C (Fig. 3). Consistent with this pattern, alleles with the highest level of recoverable PRF activity at 30 °C (>30% increase in ^{51}Cr release relative to hWT PRF) were invariably associated with atypical late-onset FHL, cancer, or aggressive viral infections. Also, the extent to which PRF activity was rescued at 30 °C appeared to be a robust predictor for the delayed onset of FHL.

Suboptimal PRF Activity and Associated Pathologies. Temperature sensitivity has been an informative tool for elucidating the molecular basis for a number of genetic diseases— for example, cystic fibrosis (Δ508 *CFTR* mutant) and Gaucher disease (exemplified by several glucocerebrosidase mutants) (27). We have shown that the temperature sensitivity of different mutations in *PRF1* can be a strong predictor of pathological outcomes as diverse as overwhelming FHL in the first months of life, an atypical form of congenital FHL, and hematological cancer appearing many years later. This relationship is supported by the strong association of clinical outcomes of individual patients and our experimental data, as well as evidence derived from the predicted structure of PRF (21–23), suggesting that some mutations with relatively minor folding abnormalities are capable of partial function in vivo. Thus, our in vitro assays that examined the function of recombinant human PRF molecules in the context of an authentic CTL may serve as a model for predicting the clinical impact of newly identified missense PRF mutations. Our work also provides a rationale for the possible development of specific drug therapies, based on stabilizing PRF structure, perhaps similar to the approach taken for therapeutic strategies of Gaucher's and Fabry disease, cystic fibrosis, and α1-antitrypsin-related emphysema (26, 28).

Our experimental data and clinical correlations establish that several mutated PRF variants can result in folding defects but still retain at least partial intrinsic cytotoxic capacity and thus have the ability to partly complement the function of CLs, should their correct folding be facilitated by endogenous factors. These variants would offer the carrier partial resistance to immune challenges and enable survival through early childhood without fulminant FHL. Similar "leaky" temperature-sensitive mutants have also been described in some other human diseases and animal models and typically result in disease of intermediate severity. As the cytotoxic activity of different PRF mutants is recovered to a variable extent at 30 °C, such an outcome becomes more likely for the product of some alleles than for others. For example, the amino acid substitutions hH222Q and hT435M are very poorly tolerated, presumably

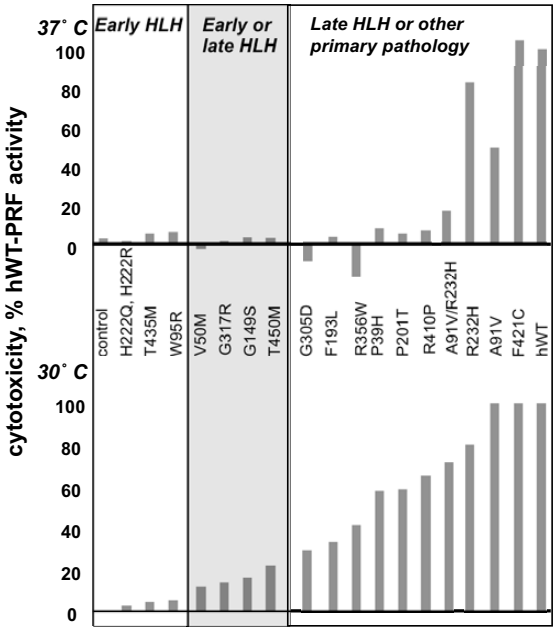


Fig. 3. The recovery of cytotoxicity by mutant PRF following culture at 30 °C correlates with the onset of FHL or other pathologies. Shown is mean relative cytotoxicity of hWT and various hPRF mutants when expressed in PRF-deficient mouse primary CTLs and grown either at 37 °C or 30 °C for 18–24 h before their use in ^{51}Cr release cytotoxicity assays, performed at 37 °C at an E/T ratio of 10. The mutants showing minimal recovery of cytotoxicity (<5% of hWT PRF levels) were invariably associated with early onset FHL. Mutants that have been consistently associated with late onset FHL or pathologies other than primary FHL had constitutive activity following culture at 37 °C or increased their cytotoxicity to >30% of hWT PRF levels when cultured at 30 °C. A further group of mutants with intermediate recovery of activity have been associated with either early or late onset FHL.

because they either produce abnormalities too severe to be overcome by chaperoning mechanisms expressed in the activated CTLs and/or because they also cause defective function at the level of the target cell. In such cases, early-onset FHL is almost invariably the outcome.

During the last several decades, a number of associative and correlative studies have attempted to address the issue of cancer immune surveillance in humans. However, to the best of our knowledge, there has been no report providing mechanistic evidence for such a link. This lack of report is perhaps unsurprising as humans appear to be highly susceptible to severe immune deficiencies, particularly those related to a dysfunctional granule-mediated pathway such as *PRF1*-dependent FHL, and generally do not survive beyond infancy without allogenic BMT. The impact of less profound immune deficiency syndromes on cancer susceptibility is difficult to interpret because of the genetic and epigenetic heterogeneity of humans. Other congenital or acquired immune deficiency disorders influence many cellular pathways or a variety of immune cell types. These considerations make the partial PRF deficiency described in this study unique in that PRF is expressed only in CL and has a single CL-specific function, namely the execution of granule-mediated cytotoxicity that is generally acknowledged to be pivotal for the elimination of infected or otherwise "dangerous" cells.

In conclusion, we contend that the combination of clinical, functional, and structural data presented here provide the most compelling evidence to date for immune surveillance of hematological cancer in humans, and a key role for PRF in that process. We therefore propose that PRF deficiency can be considered a genetic disorder of human immune surveillance.

Materials and Methods

Each patient, whose mutant *PRF1* phenotype was analyzed in the current study, was assigned with an arbitrary number from 1 to 41. All of the missense mutations reported in the current study were generated on the hWT PRF cDNA backbone by using the QuikChange site-directed mutagenesis methodology (Stratagene). Primary CTLs of *PRF1*-KO C57BL/6 mice that also express a transgenic T cell receptor (OT-1) recognizing the ovalbumin peptide SIINFEKL on EL-4 thymoma target cells (H-2K^b), were generated as described (19). Primary CTL culture, transfection using the Amaxa Nucleofector Technology, FACS sorting of transfected lymphocytes, and ^{51}Cr release cytotoxicity assays were all performed as described (19). The culture of rat basophil leukemia (RBL)-2H3 cells, gene transfection, FACS sorting, and cytotoxicity assays were also carried out as previously described (29). Western immunoblotting was performed using reducing or nonreducing Laemmli SDS/PAGE loading buffer, and PRF was detected using rat anti-mouse mAb (P1–8, provided by H. Yagita, Juntendo University, Tokyo) as described in ref. 29.

Following transfection, the cells were cultured at 37 °C in 5% CO_2 (mouse lymphocytes) or 10% CO_2 (RBL-2H3 cells) for 18–24 h. To test for temperature sensitivity, cells were cultured at 30 °C for 18–24 h after transfection. This reduced temperature had no effect on cell viability. All cytotoxicity assays were performed at 37 °C. Where indicated, the results were expressed either as: (*i*) percentage of specific ^{51}Cr release = (*test* ^{51}Cr cpm − *spontaneous* ^{51}Cr cpm)/(*total* ^{51}Cr cpm − *spontaneous* ^{51}Cr cpm) × 100, or (*ii*) percentage of relative ^{51}Cr release—the value calculated in *i* expressed as a percentage of specific ^{51}Cr release obtained with hWT PRF-transfected CTLs at the E/T ratio of 10.

Immunofluorescence microscopy was performed on RBL cells transiently transfected with mutant human PRF cDNA and grown either at 37 °C or 30 °C. The cells were fixed using 3.7% paraformaldehyde, permeabilized with 0.1% Triton, and blocked with 0.1% BSA (all dissolved in PBS, pH 7.4). The cells were probed with the primary mouse anti-human PRF (clone δG9; BD PharMingen) and the secondary fluorescent Alexa 594 antibodies (Molecular Probes).

Structural analysis of PRF amino acid substitutions was performed using the X-ray crystal structures of Plu-MACPF (PDB identifier 2QP2; ref. 22) and human complement component 8α (C8α, PDB identifier 2QQH; ref. 21). The location of PRF mutations in the MACPF fold was mapped using the published sequence alignment of MACPF proteins (22).

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1. Podack ER, Young JD, Cohn ZA (1985) Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. *Proc Natl Acad Sci USA* 82:8629–8633.
2. Trapani JA, Smyth MJ (2002) Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* 2:735–747.
3. Stepp SE, et al. (1999) Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* 286:1957–1959.
4. Molleran Lee S, et al. (2004) Characterisation of diverse PRF1 mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis. *J Med Genet* 41:137–144.
5. Henter JI, Elinder G, Soder O, Ost A (1991) Incidence in Sweden and clinical features of familial hemophagocytic lymphohistiocytosis. *Acta Paediatr Scand* 80:428–435.
6. Feldmann J, et al. (2003) Munc13–4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell* 115:461–473.
7. zur Stadt U, et al. (2005) Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. *Hum Mol Genet* 14:827–834.
8. Menager MM, et al. (2007) Secretory cytotoxic granule maturation and exocytosis require the effector protein hMunc13–4. *Nat Immunol* 8:257–267.
9. Henter JI, et al. (2007) HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer* 48:124–131.
10. Walsh CM, et al. (1994) Immune function in mice lacking the perforin gene. *Proc Natl Acad Sci USA* 91:10854–10858.
11. Koebel CM, et al. (2007) Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450:903–908.
12. Willimsky G, Blankenstein T (2007) The adaptive immune response to sporadic cancer. *Immunol Rev* 220:102–112.
13. Smyth MJ, et al. (2000) Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *J Exp Med* 192:755–760.
14. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: From immunosurveillance to tumor escape. *Nat Immunol* 3:991–998.
15. Galon J, et al. (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313:1960–1964.
16. Voskoboinik I, Smyth MJ, Trapani JA (2006) Perforin-mediated target-cell death and immune homeostasis. *Nat Rev Immunol* 6:940–952.
17. Ries LAG, et al. (2008) Surveillance, Epidemiology, and End Results (SEER) Program (National Cancer Institute, Bethesda, MD). Available at http://seer.cancer.gov/csr/1975 2005.
18. Gatti RA, Good RA (1971) Occurrence of malignancy in immunodeficiency diseases. A literature review. *Cancer* 28:89–98.
19. Voskoboinik I, et al. (2007) Perforin activity and immune homeostasis: The common A91V polymorphism in perforin results in both presynaptic and postsynaptic defects in function. *Blood* 110:1184–1190.
20. Voskoboinik I, Thia MC, Trapani JA (2005) A functional analysis of the putative polymorphisms A91V and N252S and 22 missense perforin mutations associated with familial hemophagocytic lymphohistiocytosis. *Blood* 105:4700–4706.
21. Hadders MA, Beringer DX, Gros P (2007) Structure of C8alpha-MACPF reveals mechanism of membrane attack in complement immune defense. *Science* 317:1552–1554.
22. Rosado CJ, et al. (2007) A common fold mediates vertebrate defense and bacterial attack. *Science* 317:1548–1551.
23. Slade DJ, et al. (2008) Crystal structure of the MACPF domain of human complement protein C8 alpha in complex with the C8 gamma subunit. *J Mol Biol* 379:331–342.
24. Baran K, et al. (2009) The molecular basis for perforin oligomerization and transmembrane pore assembly. *Immunity* 30:684–695.
25. Whisstock JC, Bottomley SP (2006) Molecular gymnastics: Serpin structure, folding and misfolding. *Curr Opin Struct Biol* 16:761–768.
26. Mu TW, et al. (2008) Chemical and biological approaches synergize to ameliorate protein-folding diseases. *Cell* 134:769–781.
27. Wiseman RL, Powers ET, Buxbaum JN, Kelly JW, Balch WE (2007) An adaptable standard for protein export from the endoplasmic reticulum. *Cell* 131:809–821.
28. Cohen FE, Kelly JM (2003) Therapeutic approaches to protein-misfolding diseases. *Nature* 426:905–909.
29. Voskoboinik I, et al. (2004) The functional basis for hemophagocytic lymphohistiocytosis in a patient with co-inherited missense mutations in the perforin (*PFN1*) gene. *J Exp Med* 200:811–816.
30. Clementi R, et al. (2005) A proportion of patients with lymphoma may harbor mutations of the perforin gene. *Blood* 105:4424–4428.
31. Cannella S, et al. (2007) Germline mutations of the perforin gene are a frequent occurrence in childhood anaplastic large cell lymphoma. *Cancer* 109:2566–2571.
32. Clementi R, et al. (2002) Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying PRF1 mutations. *Blood* 100:2266–2267.
33. Santoro A, et al. (2005) A single amino acid change A91V in perforin: A novel, frequent predisposing factor to childhood acute lymphoblastic leukemia? *Haematologica* 90:697–698.
34. Mancebo E, et al. (2006) Familial hemophagocytic lymphohistiocytosis in an adult patient homozygous for A91V in the perforin gene, with tuberculosis infection. *Haematologica* 91:1257–1260.
35. Mehta PA, et al. (2006) Perforin polymorphism A91V and susceptibility to B-precursor childhood acute lymphoblastic leukemia: A report from the Children's Oncology Group. *Leukemia* 20:1539–1541.
36. Beaty AD, et al. (2007) A teenage boy with late onset hemophagocytic lymphohistiocytosis with predominant neurologic disease and perforin deficiency. *Pediatr Blood Cancer* 50:1070–1072.
37. Katano H, et al. (2004) Chronic active Epstein–Barr virus infection associated with mutations in perforin that impair its maturation. *Blood* 103:1244–1252.
38. zur Stadt U, et al. (2006) Mutation spectrum in children with primary hemophagocytic lymphohistiocytosis: Molecular and functional analyses of PRF1, UNC13D, STX11, and RAB27A. *Hum Mutat* 27:62–68.
39. Ishii E, et al. (2005) Genetic subtypes of familial hemophagocytic lymphohistiocytosis: Correlations with clinical features and cytotoxic T lymphocyte/natural killer cell functions. *Blood* 105:3442–3448.
40. Busiello R, et al. (2004) Atypical features of familial hemophagocytic lymphohistiocytosis. *Blood* 103:4610–4612.
41. Feldmann J, et al. (2002) Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. *Br J Haematol* 117:965–972.